Multivalent Ligand Displayed on Plant Virus Induce Rapid Onset of Bone Differentiation

*L. Andrew Lee, Sevan M. Muhammad, Quyen Nguyen, Pongkwan Sitasuwan, Gary Horvath, Qian Wang**

University of South Carolina, Department of Chemistry and Biochemistry
631 Sumter St. Columbia, SC 29208, E-mail: wang@chem.sc.edu.
Homepage: www.chem.sc.edu/faculty/wang/

Materials and Methods:

Layer by layer assembly and substrate characterization: For tissue culture plasticware preparation, the 6-well plate was cleaned with O_2 plasma cleaner for 15 minutes. The first coating was for 30 minutes with poly(allylamine) hydrochloride (PAH, Mw ~ 56,000, Aldrich) at a concentration of 1 mg/mL in 150 mM NaCl, filtered through 0.2 micron membrane (PALL). The wells were washed three times with dH_2O (Millipore 18.2 MΩ) to remove unbound polyelectrolytes. Then each wells were incubated for 15 minutes with 1 mL of poly(styrene sulfonate) (PSS, Mw ~ 70,000, Sigma) at a concentration of 2 mg/mL in 150 mM NaCl, filtered through 0.2 micron membrane (PALL). The polyelectrolyte solution was removed and wells were washed three times with water. The coating of polyelectrolytes, alternating between PAH and PSS, was repeated until a total of 7 layers were coated. The final layer was then coated with TMV or TMV-RGD1 (prepared at 1 mg/mL and filtered through 0.45 micron HT Tuffryn Membrane Syringe Filter, PALL life sciences). The final coating was washed with water three times and dried overnight. Prior to seeding the cells, the substrates were treated with UV irradiation for 15 minutes. All substrates were prepared within one day prior to seeding of cells.

For quartz crystal microbalance measurements, the silver electrodes were washed three times with dH_2O (18.2 MΩ), dried under a stream of N_2. The dried electrodes were measured (9 MHz). The electrodes were then coated with PAH for 30 minutes at room temperature. The electrodes were washed with water and then dried under a stream of N_2. The dried electrodes were measured and the shift in oscillation frequencies was recorded. The coating, washing, and drying steps were repeated as each new layer of polyelectrolytes was added to the silver electrodes. All measurements were conducted in triplicates and in three separate experiments. The frequency shifts were converted to mass densities using Sauerbrey's equation.

$$\Delta f = \frac{-2\Delta m f_o^2}{A\sqrt{\rho_q \mu_q}}$$

Uncoated PEM and TMV coated PEM substrates were further characterized by water contact angle measurements. The piranha solution treated microscope coverslips (VWR 18 mm diameter, No. 2 thickness) measured approximately 10° ± 1° and the final PEM averaged 28° ± 5°. Coating of the virus did not alter the water contact significantly, but could be well visualized by atomic force microscopy and mass density shift (QCM).

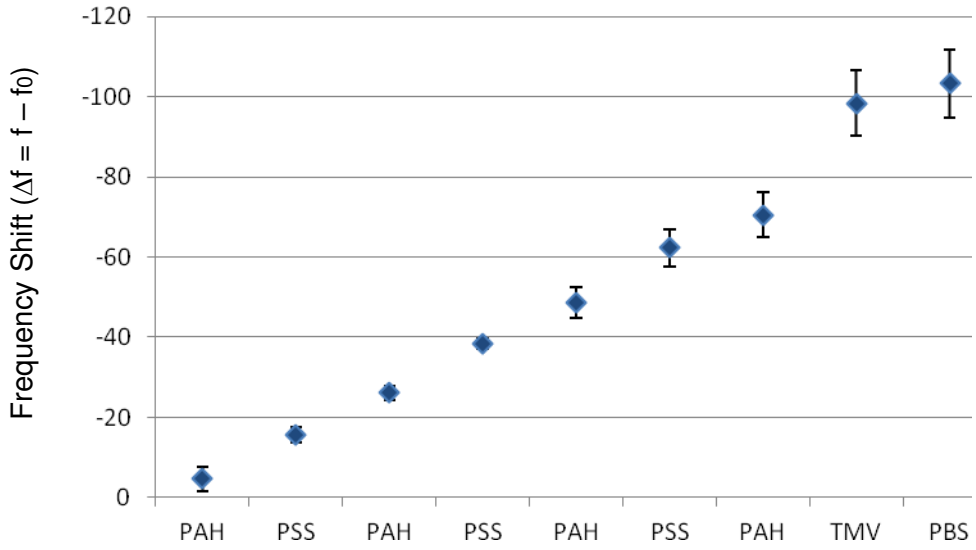
Cell culture: Rat bone marrow stromal cells (rBMSCs) were harvested according to protocol approved by IACUC. Passages from 5-8 were used in the study. Rat BMSCs were isolated from the bone marrow of young adult male Wister rats (80 g, Harlan Sprague Dawley, Inc.). The procedures were performed in accordance with the guidelines for animal experimentation by the Institutional Animal Care and Use Committee, University of South Carolina. The isolated BMSCs were maintained and expanded for an additional passage in DMEM with 10% FBS. All cell culture reagents were purchased from HyClone. BMSCs were induced with DMEM/F12 without serum and had been supplemented with osteogenic factors (10 nM dexamethasone, 50 μg mL^{-1} ascorbic acid, 10 mM β-glycerophosphate) and growth factors

(10 ng mL^{-1} recombinant human bFGF, 2.5 ng mL^{-1} recombinant human TGF-β, 10 ng mL^{-1} recombinant human EGF) and 1x ITS (insulin, transferrin, selenium). Dexamethasone, ascorbic acid and β-glycerophosphate were purchased from Sigma-Aldrich. Recombinant human bFGF and EGF were purchased from Stemgent, recombinant human TGF-β was purchased from StemRD. 100x ITS stock was purchased from VWR/Mediatech. The cells were cultured to near 80% confluence and harvested by trypsinization. 4 mL of 0.25% Trypsin/EDTA (Hyclone) was used to dislodge the cells from the flasks and $3x10^5$ cells were seeded in each well.

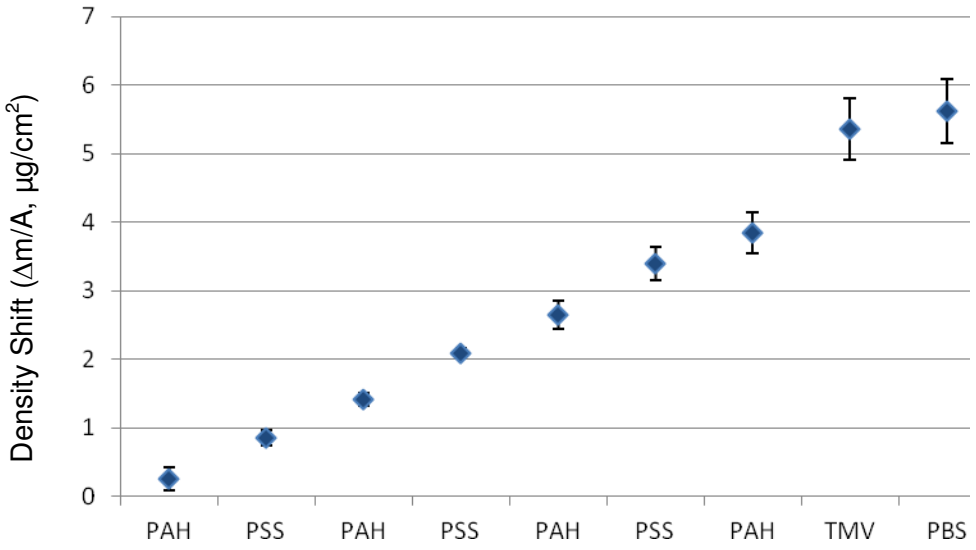
Cytochemical Staining and Immunofluorescence: Batches of BMSCs culture on TMV coated substrates were terminated post 2 days of osteoinduction. Cells were fixed in 4% PFA in 1x D-PBS pH 7.4 at room temperature for 15 minutes. Each of the samples was then permeablized for 5 minutes with 1 x D-PBS, 0.05% Triton X-100 and blocked with 3% bovine serum albumin (BSA, Rockland), 1x D-PBS for 1 hour at room temperature. After blocking, the cells were incubated overnight with primary antibodies targeting the osteo-specific marker osteocalcin. The secondary antibodies goat anti-rat FITC (VWR Scientific) were used for osteocalcin (*BGLAP)* at 1:100 dilutions in buffer at 4°C. TRITC-phalloidin (1:200 in 1x D-PBS) was used to stain filamentous actin. Nuclei were stained with DAPI (4, 6-diamidino-2-phenylindole, 100 ng/ml). Images of the stained substrates were collected using Olympus IX81 fluorescent microscope with 60x oil lens (UPlanSAPO, NA = 1.35). Negative control for staining included only secondary antibodies. After 2 days in osteogenic cultures, BMSCs seeded on hFN, PEM, TMV and TMV-RGD1 substrates were stained with Alizarin red calcium rich deposits. The cells were fixed in 4% paraformaldehyde at room temperature for 5 minutes, washed with water and then stained with 0.1% solution of Alizarin red (Sigma-Aldrich) pH 4.1-4.5 for 60 minutes at room temperature. Since the reaction was highly light sensitive, the substrates were wrapped in aluminum foil during the entire time of incubation.

ELISA and Western Blot: Cells were fixed in 4% paraformaldehyde/1x D-PBS for 5 minutes at room temperature. The cells were washed and blocked with 1% BSA/1x D-PBS/0.05% Triton X-100 for 30 minutes. The primary antibody against BMP-2 was incubated in three wells overnight at 4 degrees celsius at a dilution of 1:100. The samples were rinsed with PBS, 0.05% Triton X-100, three times and the secondary antibody (anti-mouse Goat polyclonal with HRP conjugate) was incubated for 1 hour at room temperature. The samples were washed three times and incubated with TMB solution for 30 minutes and the reaction was stopped with concentrated sulfuric acid. The solution was read with a UV-Vis spectrophotometer at 450 nm.

For western blots, cells were cultured on 6-well plates. The media was aspirated and the cells were directly lysed by adding 100 µl of Laemmeli buffer (Bio-rad) containing 2-mercaptoethanol and protease inhibitor, PMSF (1 µg/mL). The lysate was scrapped and transferred to a clean microfuge tube and heated at 95°C for 15 minutes, then immediately cooled on ice prior to loading on a 12% SDS-PAGE. The gel was run for 60 minutes at 200 V in a Mini-Protean 3 Gel electrophoresis rig (Bio-rad). The gel was transferred to PVDF membrane then block with 2% BSA in PBS with 0.05% Triton X-100 (PBS-T) for one hour at room temperature. The primary antibody (total FAK or pY397 FAK, Cell Signaling Technology) was added to the block solution at 1:1000 dilution and incubated overnight. The membrane was washed three times with PBS-T, then probed with secondary antibody (anti-rabbit goat IgG with HRP conjugate) at 1:5000 dilution for one hour at room temperature in 2% BSA in PBS-T. The membrane was washed three times, then imaged with enhanced chemiluminesence kit (Pierce ECL) according to manufacturer's protocol. The resulting blots were scanned and intensity plots were measured by ImageJ. The average of three separate images were used. The intensity of pY397 FAK was normalized to total FAK for all samples and the normalized intensities were plotted relative to cell lysates from hFN substrates.



Supplementary Figure 1. QCM measurements of PAH/PSS polyelectrolyte membrane with TMV. Each layer of coating decreases the frequency in a linear fashion. TMV coating was followed by incubation in 1x PBS solution for one hour. A slight shift in frequency was observed, likely due to the osmolarity difference between TMV (100 mM K Phos pH 7.0) versus PBS. Error bars denote standard deviation from the mean (n = 9) and 3 separate experiments in triplicates.



Supplementary Figure 2. Mass density shift of polyelectrolyte membrane. A linear mass shift is observed until TMV coating, where the total amount of viral particles averaged 1.5 ± 0.3 µg/cm^2. Incubation with PBS solution allowed for slight swelling of the PEM, increasing the density shift by 0.3 ± 0.1 µg/cm^2. Error bars denote standard deviation from the mean (n = 9) from three separate experiments performed in triplicates.